IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of

Jiro HITOMI, et al. GROUP: 1644

SERIAL NO.: 09/910,208 EXAMINER: Maher M. Haddad

FILED: July 20, 2001

FOR: NOVEL CALCIUM-BINDING PROTEINS

Commissioner for Patents
P.O.Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 CFR 1.132

Sir:

I, Jiro Hitomi, declare as follows:

1. I hold a *(type of degree(s))* Doctor of Medicine degree(s) from *(university)* Niigata University Postgraduate School of Medicine in *(subject(s))* Pathology.

2. I have been employed by Iwate Medical University as (and have the title of) Professor since 2003 in the madical department. I have direct experience in Anatomy and Molecular biology and am capable of rendering an opinion as an expert in the art of biotechnology and immunology.

3. I am a joint inventor of the above identified patent application and have read the outstanding rejection under 35 USC 112, first paragraph, for lack of enablement in the Official Action dated May 31, 2006.

4. I have reread the specification of the subject patent application and all of the arguments raised by the Examiner during the prosecution.

5. The following experiments were performed under my supervision and direction:

A. Comparison of hCAAF1 Levels in Cancerous and Non-Cancerous Sites of Esophageal Tissue by ELISA

i. Materials and Methods

a. Preparation of Human Esophageal Tissue Samples

Six surgically excised samples of esophageal **tissue** were divided into cancerous and non-cancerous sites. 2 mL of HEPES buffer (pH 7.4) containing 1 mM EDTA, 1 mM DTT and complete protease inhibitor were added to about 200 µg of each tissue section followed by homogenizing using a Teflon homogenizer. The supernatant obtained by centrifuging for 10 minutes at 4°C and 2000 rpm was additionally centrifuged for 60 minutes at 4°C and 30,000 rpm, and the resulting supernatant was used as sample. The protein concentration in each sample was measured using a Bradford Protein Assay Kit (BioRad).

b. hCAAF1 ELISA

Anti-human CAAF1 monoclonal antibody hCF128 was diluted to a final concentration of 0.3 µg/mL with 0.1 M sodium carbonate buffer (pH 9.6) containing 0.5 M NaCl, followed apportioning 100 µl aliquots thereof into each well of a 96-well microplate (Nunc). After allowing to stand undisturbed overnight at 4°C, the microplate was washed twice using 0.35 mL of 10 mM sodium phosphate buffer (pH 7.3) containing 0.15 M NaCl, followed by the addition of

0.35 mL of 20 mM HEPES buffer (pH 7.4) containing 1% BSA and 0.15 M NaCl (to be referred to as a blocking solution), and allowing to stand undisturbed for 2 hours at room temperature.

After removing the blocking solution, 100 μL of 20 mM HEPES buffer (pH 7.4) containing 1% BSA, 1 mM EDTA and 0.05% Tween 20 and 10 μL of measurement sample were added to each well and allowed to react for 1 hour at room temperature. The microplate was washed five times with 0.35 mL of HEPES buffer (pH 7.4) containing 0.05% Tween 20 (washing solution) followed by the addition of 100 μL of standard antibody solution diluted with HEPES buffer (pH 7.4) containing 1 mM $CaCl_2$, 1% BSA and 0.05% Tween 20 to a concentration of peroxidase (POD)-labeled monoclonal antibody (hCF113) of 0.1 μg/mL, and allowing to react for 1 hour at room temperature. Following the reaction, the microplate was washed six times with 0.35 mL of the aforementioned washing solution followed by the addition of 100 μL of substrate solution (ortho-phenylenediamine) and reacting for 30 minutes at room temperature. 100 μl of 1 M sulfuric acid solution were added followed by measurement of optical absorbance at a wavelength of 492 nm (OD 492) using the optical absorbance at a wavelength of 630 nm as a control.

The concentration of hCAAF1 in esophageal tissue was calculated from a calibration curve prepared using recombinant human CAAF1 (rhCAAF1), and the concentration was converted to the amount of hCAAF1 in units of ng/mg protein based on the results of quantifying the amount of protein in each sample.

ii. Results

As shown in Fig. 1, cancerous sites demonstrated higher levels of hCAAF1 per amount of tissue protein than non-cancerous sites in 4 of 6 cases of esophageal cancer. **The levels of hCAAF1 in cancerous to non-cancerous sites ranged from about 2:1 in sample no. 10834 to about 8:1 in sample no. 11300.**

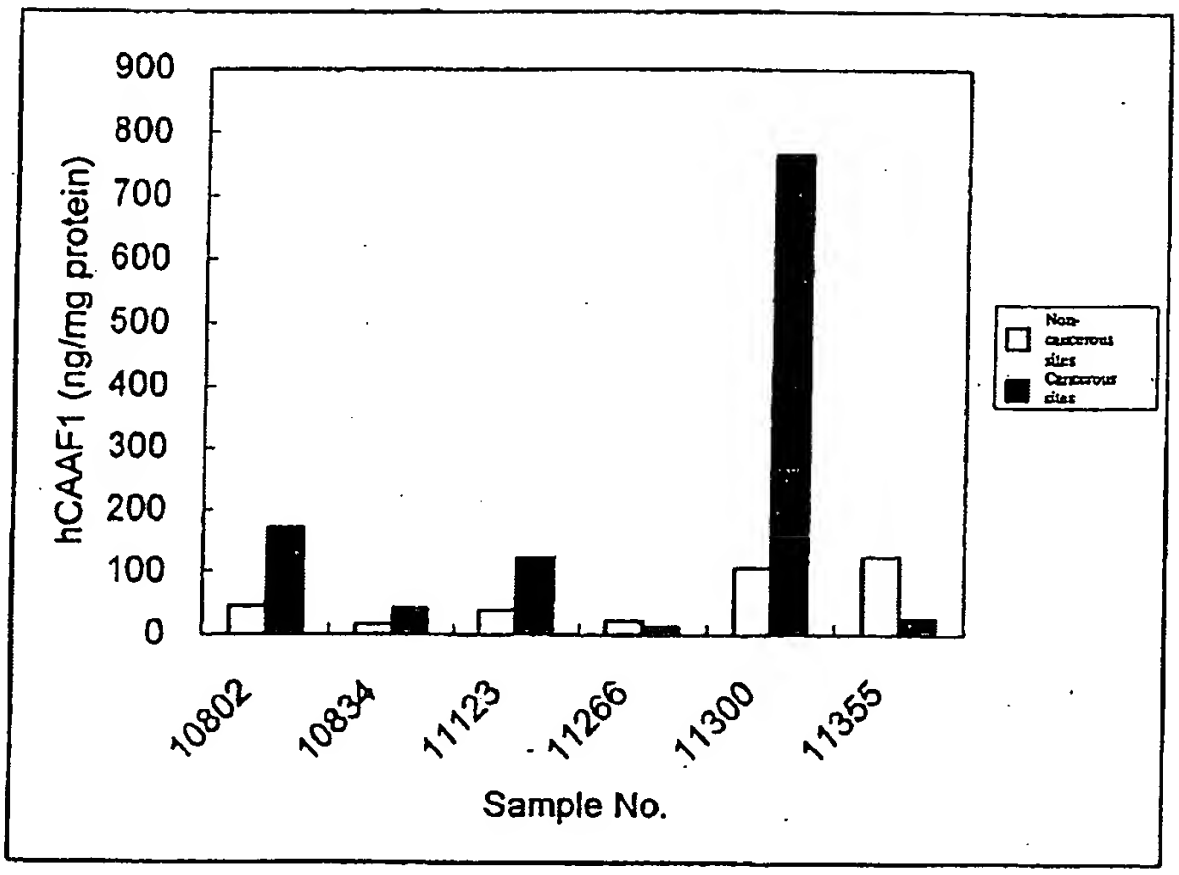

Fig. 1 Comparison of hCAAF1 Levels in Cancerous and Non-Cancerous Sites of Esophageal Tissue

B. Expression of CAAF1 in Normal and Diseased Skin Tissue by Immunohistostaining

i. Materials and Methods

Surgical samples of each skin tissue indicated in Fig. 2 and the table were fixed with 10% formalin solution followed by the preparation of paraffin-embedded sections for use in immunohistostaining.

CAFF1-22-5, which is a bovine CAFF1 mouse monoclonal antibody confirmed to exhibit reactivity with human CAAF1 and but not react with other proteins of the S100 family, was used for the anti-CAAF1 antibody. Mouse MoAb, clone SH-L1 (Sigma) was used for the anti-S100A2 (S100L) antibody, while mouse MoAb, clone CACY-100 (Sigma) was used for the anti-S100A6 (calcyclin) antibody. Alkaline phosphatase-labeled goat anti-mouse Ig polyclonal antibody was used for the secondary antibody, and developed with Fast Red.

ii. Results

As shown in the photos of Fig. 2, although strongly positive reactions were observed for CAAF1 at the affected sites in all four cases analyzed by immunostaining in Bowen's disease of squamous cell carcinoma (SCC in situ) and squamous cell carcinoma of the skin, expression of S100A2 and S100A6 was not observed. With respect to other skin diseases, there were many cases in which expression **of CAAF1** was observed in inflammatory diseases and epidermal tumors as shown in the table **by a positive (+) or strongly positive (++) designation**.

C. Expression of CAAF1 in Inflammatory Bowel Disease by Immunohistostaining

i. Materials and Methods

Surgical samples of ulcerative colitis tissue were fixed in 10% formalin solution followed by the preparation of paraffin-embedded sections for use in immunohistostaining.

The bovine CAAF1 monoclonal antibody, CAAF1-22-5, was used for the anti-CAAF1 antibody. HRP-labeled goat anti-mouse Ig polyclonal antibody was used for the secondary antibody, and developed with DAB. HE (hematoxylin-eosin) staining was carried out according to routine methods.

ii. Results

As shown in Fig. 3, in cases of inflammatory bowel disease in the form of ulcerative colitis, expression of CAAF1 was observed at erosion sites around the ulcerations (Fig. 3, top). In addition, strongly positive reactions were observed for CAAF1 at sites where necrosis was occurring at the sites of ulceration (Fig. 3, bottom).

D. Expression of CAAF1 in Polymorphonuclear Leukocytes (PMN) by Immunohistostaining

i. Materials and Methods

Surgical samples of PMN were fixed with 10% formalin solution followed by the preparation of paraffin-embedded sections for use in immunohistostaining.

The bovine CAAF1 monoclonal antibody, CAAF1-22-5, was used for the anti-CAAF1 antibody. Alkaline phosphatase-labeled goat anti-mouse Ig polyclonal antibody was used for the secondary antibody and developed with Fast Red.

ii. Results

Although PMN are activated as a result of stimulation by migration factor and adhere to vascular endothelium, PMN adhered to human subcutaneous vascular endothelium were strongly positive for CAAF1. In addition, perivascular tissue to which PMN were adhered was also positive for CAAF1 (Fig. 4). It is presumed that adhered neutrophils released CAAF1 and that CAAF1 molecules were also scattered around the vessels. On the basis of this image, PMN producing CAAF1 can be expected to have the potential to cause fluctuations in expression of CAAF1 as a result of abnormalities occurring in the PMN themselves. The triangles indicate PMN, while the arrows indicate vascular endothelium.

E. Relationship of CAAF1 Expression in PMN to macrophages and other similar lineages

The foregoing results provide proof that not only PMN, but macrophages and other cell types along these lineages would be expected to express CAAF1. As persons skilled in the art know, PMN, macrophages and other cell types along these lineages share common characteristics

related to white blood cells. Accordingly, by showing that PMN express CAAF1 as evidenced by Fig. 4, it has, in fact, been established the expectation that macrophages and other cell types along these lineages will express CAAF1.

6. Based upon my experiment(s) which demonstrate enablement, I respectfully request that the rejection under 35 USC 112, first paragraph, for lack of enablement to be withdrawn.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed By: Jiro Hitomi

Jiro Hitomi

Dated: Feb. 26, 2008